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13. ABSTRACT ***(Maximum 200 Words)***

The proposed work focuses on identifying the genes and their function that are involved in the acquisition of peak bone density and also the genes and their function that regulate the anabolic skeletal response to mechanical stress. To gain information about genes regulating bone density, we have identified three large pedigrees in which there are subjects with very high bone density. The long term goal will be to perform genome-wide linkage analyses to ultimately identify the gene (and its corresponding function) responsible for the high bone density in each of the three pedigrees. The proposed work on mechanical strain involves a QTL anaylsis of mouse periosteal bone formation in response to jump training. During the first twelve months of the funding period, we proposed several technical objectives for the studies mentioned above. As disclosed in this progress report, we have successfully accomplished all of the specific objectives outlined for the first award year in addition to completing many additional objectives above and beyond those specific objetcives outlined in the original proposal.

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General Introduction

It is well established that genetic factors are important determinants of bone density, risk of fracture, and the body's anabolic skeletal response to mechanical stress. However, until recently, little has been known about the specific genetic factors that exert dominant effects on bone density and anabolic skeletal response to mechanical strain.

The work proposed in this study focuses on identifying those genes and the functions thereof involved in the acquisition of peak bone density, as well as the genes responsible for regulating the body's anabolic skeletal response to mechanical stress. The importance of this work is that while most current therapeutic options target prevention of bone loss through the inhibition of bone resorption, our effort focuses on identifying those genetic factors and biological pathways related to bone formation itself, enabling us to suggest new strategies for bone loss prevention and therapy.

Until recently, detecting the influence of any given gene on a complex trait was an arduous task at best. Due to polygenic and polyenvironmental determinants of the acquisition of peak bone density, as well as the anabolic skeletal response to mechanical stress (these quantitative traits are also referred to as complex traits), the influence of any single gene on these complex traits must be detected amid considerable "noise" from both other genetic factors and non-genetic sources of variation. However, through the implementation of new technologies such as linkage mapping using whole genome scan, microarray, and QTL mapping using different strains of mice, it is now possible to determine genetic influence on complex traits such as bone density and anabolic skeletal response to mechanical loading. Therefore, to accomplish our goal, we have proposed two major approaches: linkage studies using large human pedigrees with high bone density, and QTL mapping using different mouse strains.

Our goals for the first twelve months of the funding period for each of the technical objectives, as well as our progress to date for each technical objective, are described below. The progress report for each technical objective is organized according to the outline provided by the office of the U.S Army Medical Research and Materiel Command.

In addition to completing all of the technical objectives required for the grant period, we are reporting progress on additional objectives accomplished above and beyond those contracted for. These additional objectives are identified as "Additional Progress" in the report.

1. Molecular Genetic Studies of Bone Mechanical Strain

Introduction

Increased bone formation in response to loading is a significant factor in controlling loss of bone with age and in disease conditions. Studies of mechanical loading in two different strains of mice have revealed differential types of phenotypes in relation to bone formation. The B6 strain (Low Bone Density) showed a high response to mechanical loading while the C3H strain (High Bone Density) was less responsive. The goal of our research program is to investigate the genes that are expressed in mechanical loading for QTL analysis. Accordingly, we undertook experiments to optimize a specific type of mechanical loading that would provoke the change in the bone cell phenotype that would lead to expression of target genes and, consequently, bone formation. In addition,

we also determined the optimal quantity of RNA for the present and future analysis of gene expression using Microarray and real time PCR.

A. Technical Objective 1:

Our goals for this Objective are to perform jump training in 10 different mouse strains and to evaluate the bone formation response to this jump training in order to identify two mouse strains that show extreme differences in their bone formation response to mechanical loading. These two strains will then be applied for QTL studies and for *in vitro* studies that will be used to identify candidate genes responsible for mediating the bone formation response to mechanical loading. If during the course of these jump studies we find that the jump training technique does not produce a phenotype equivalent in magnitude to the 4-point bending phenotype (i.e., one which causes a large increase in bone formation), we will shift our efforts to the 4-point bending procedure for providing an *in vivo* mechanical strain. The jump training is the preferred method because it is more physiological than the 4-point bending procedure.

This report includes our progress in these studies for the last twelve months of our proposal, with results for these specific objectives listed in the order outlined in our proposal. This order is as follows:

1) Develop the conditions for optimal jump training technique, which include:
    a) Determining duration of the study (i.e., between 7 and 28 days);
    b) Evaluating the most sensitive endpoints, which include histomorphometric measurements of bone formation, pQCT measurements of the bone perimeter and/or serum biochemical markers of bone formation;
    c) Determining the optimal sampling site in the skeleton (i.e., the tibia or the femur);
    d) Determining if the entire bone perimeter should be evaluated or whether only the cortical mid-shaft is required for a sensitive assay.
2) Select the optimal mouse pair for our QTL studies. We will also use this mouse pair for our *in vitro* studies designed to discover candidate genes that might mediate the bone formation response to mechanical loading in vitro.

To date, we have accomplished most of the specific objectives cited above. Our progress in each of these specific objectives is given below.

Progress on Technical Objective 1

In this study, five to six week old mice, six-to-seven mice per group, were evaluated after two, three and four weeks of jump training, respectively. Each mouse jumped ten times per day, five days a week, to a height of 25 centimeters. Control mice were placed in the same cage but were not given the electrical stimulation to jump. The end points of this study were pQCT parameters of the femur and serum biochemical markers. The precision of the pQCT was less than 3% and the precision of the biochemical markers (i.e. mouse C-telopeptide, mouse osteocalcin and mouse skeletal alkaline phosphatase) was 7%, 10% and 2%, respectively, within the assay CV. In the pQCT study, we scanned the femur nine times, covering the entire length of the bone. These data are presented as the midshaft slice for all of the density and area calculations.

We also examined all nine slices to determine if there was any one site more sensitive to the jump training than the others.

Specific Objective 1: After the optimum study duration (two weeks of jump training), no significant changes in pQCT parameters or biochemical markers were evident. At three weeks of jumping, the changes were maximal with significant increases in periosteal parameter, cortical thickness and cortical area (**Tables 1, 2 & 3**). However, at four weeks, while significant changes were still seen, these changes, in general, were less than those at three weeks. In addition, at three weeks, we observed significant changes in total bone mineral density. In contrast, no significant changes were seen in serum biochemical parameters at any sampling time from 2,3 and 4 weeks (**Table 4**).

Table 1. Cross-sectional parameters and area of mid-shaft femur

Parameters	2-Week Control (Mean±SD)	2-Week Jump (Mean±SD)	3-Week Control (Mean±SD)	3-Week Jump (Mean±SD)	4-Week Control (Mean±SD)	4-Week Jump (Mean±SD)
Periosteal Circumference (mm)	4.23±0.08	4.24±0.052	4.22±0.08	4.35±0.083*	4.19±0.04	4.33±0.11*
Endosteal Circumference (mm)	3.27±0.06	3.23±0.08	3.22±0.06	3.27±0.10	3.17±0.06	3.25±0.103
Cortical thickness (mm)	0.15±0.01	0.16±0.006	0.16±0.003	0.17±0.007**	0.16±0.01	0.17±0.005#
Total Area (mm^2)	1.43±0.05	1.43±0.033	1.42±0.05	1.50±0.059*	1.40±0.03	1.48±0.075*
Cortical Area (mm^2)	0.53±0.03	0.60±0.019	0.59±0.013	0.66±0.026**	0.59±0.03	0.64±0.028

p-values, *p<0.05, **p<0.01

Table 2. Bone mineral content and bone density parameters of mid-shaft femur

Parameters	2-Week Control (Mean±SD)	2-Week Jump (Mean±SD)	3-Week Control (Mean±SD)	3-Week Jump (Mean±SD)	4-Week Control (Mean±SD)	4-Week Jump (Mean±SD)
Cortical content (mg/slice)	0.69±0.04	0.72±0.02	0.71±0.03	0.80±0.02**	0.71±0.01	0.78±0.04*
Total content (mg/slice)	0.65±0.05	0.69±0.03	0.68±0.04	0.76±0.04**	0.69±0.02	0.76±0.03**
Total bone density	409±30	434±6	425±19	461±8**	445±13	465±17*

(mg/cm^3)

p-values, *p<0.01, **p<0.001

Table 3. P-values for ANOVA

	ANOVA treatment	ANOVA Weeks	Post Hoc Week 2 vs week 3 or week 4	Post Hoc Week 3 vs week 4
Periosteal Circumference	P=0.046	NS	NS	NS
Total Content	P=0.03	P=0.01	P<0.014	NS
Total Density	P=0.004	P=0.03	P<0.03	NS

Table 4. Biochemical parameters in jump exercise group compared to control group of mice.

Parameters	2-Week		3-Week		4-Week	
	Control (Mean±SD)	Jump (Mean±SD)	Control (Mean±SD)	Jump (Mean±SD)	Control (Mean±SD)	Jump (Mean±SD)
Skeletal ALP (U/L)	152± 12	141± 34	175 ± 15	191 ± 24	164 ± 23	166 ± 23
Osteocalcin (ng/ml)	166 ± 32	168 ± 46	237 ± 60	200 ± 23	170 ± 58	141 ± 20
C-telopeptide (ng/ml)	4.4 ± 1.4	5.9 ± 1.8	5.2 ± 1.4	6.0 ± 1.7	6.2 ± 1.2	6.1 ± 1.8

Differences between control and jump groups were not statistically significant.

Sensitivity of the various end points and the changes recorded with pQCT were barely significant, but not large. Therefore, histomorphometric studies were not performed because the precision of these measurements is less than that seen with pQCT. Since the biochemical markers did not show a significant change, the most sensitive test was the pQCT.

With respect to the optimal sampling site in the skeleton, we examined nine different slices throughout the femur and found, in essence, no single site that was superior to another. Therefore, we focused our efforts on the midshaft of the femur, which was slice five (**Figure 1**).

Sampling with the pQCT was superior for the femur as opposed to the tibia therefore the tibia was evaluated.

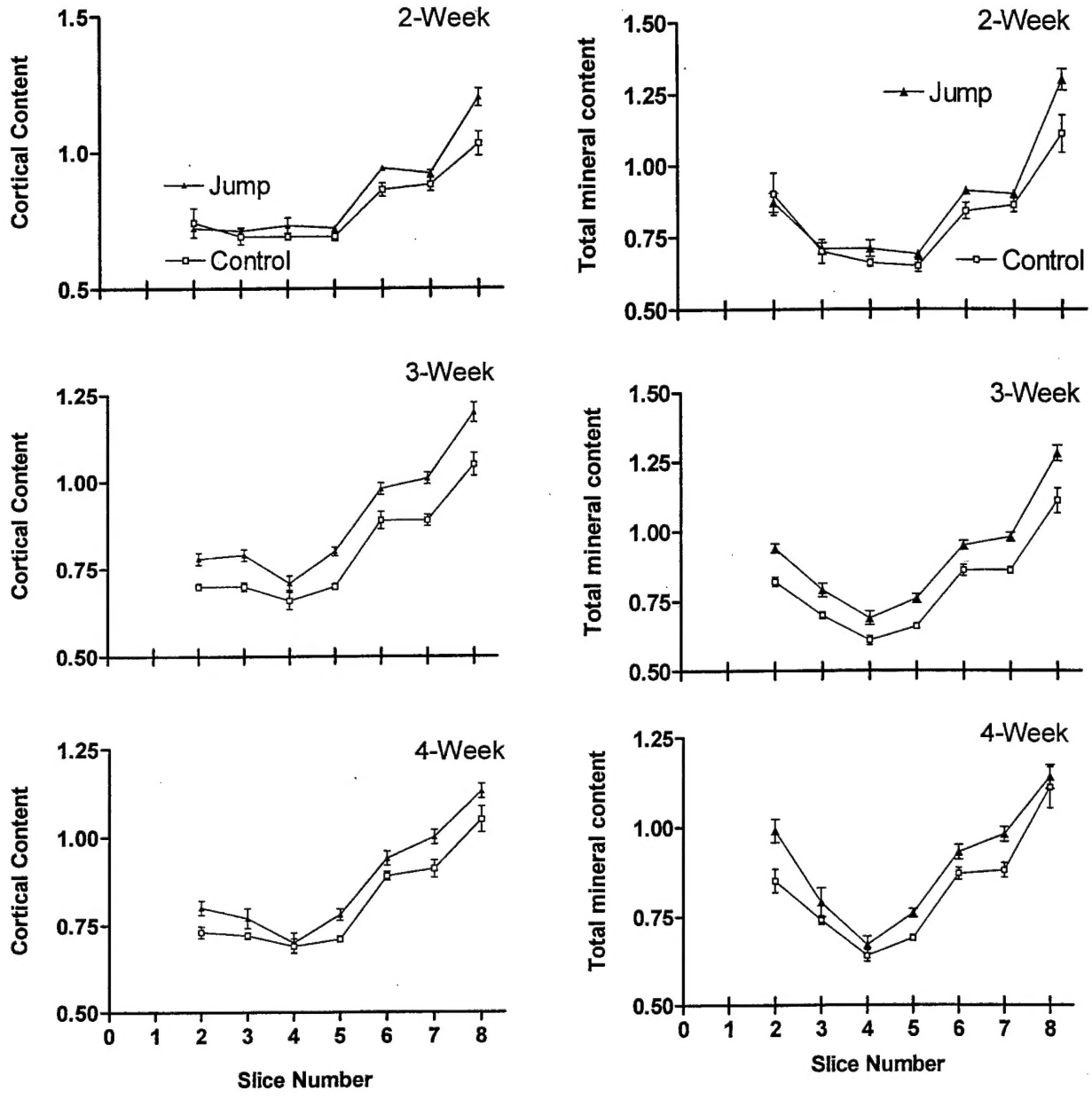


Figure 1. Effect of 2, 3 and 4 weeks of jump exercise on cortical and total bone mineral content (mg/slice) measured by pQCT at 9 different slices of the femur. Each slice was separated by 11% of the femur length, with slice 1 starting at distal end. The data shows (slices 1 & 9 omitted due to large variation) that increase in bone mineral content was throughout the length of the femur. Each data point represents mean $\pm$ standard error of mean (n=6). P-value for both cortical and total bone mineral content of control vs. jump group was <0.0001 by ANOVA for 3-week and 4-week jump exercise groups. P-value for comparison of individual slices in control and jump groups of mice by post hoc tests are #p>0.05, *p<0.05.

Specific Objective 2: Using *in vitro* strain studies, we found that the least responsive mouse strain was the C3H and the most responsive mouse strains were both the Balb-C and the B6. These mouse strains will be further evaluated for the QTL studies. The corresponding QTL experiments mentioned in Specific Objective 1 will be carried out using these mouse strains after validating with real time PCR and the 4-point bending technique mentioned above.

In conclusion, we found that the 14 day time point was optimal for jump studies using pQCT parameters as end points. The most sensitive end point was the pQCT and the optimal sampling site was the femur and, within the femur, the midshaft site appeared to be the most optimal and convenient site. While the changes with the pQCT were highly significant, they were not large enough to apply to QTL studies, where each individual animal must be assigned a quantitative phenotype. Therefore, we have made two changes in the protocol: 1) we have elected to use 4-point bending as opposed to jumping as our strain stimulus. We indicated that this approach might be necessary in the original grant application; and 2) we elected to explore the possibility of using microarray to identify genes that were markedly increased or decreased with either jump training or 4-point bending. As shown below under Additional Progress, a number of genes were found to express in bone, which made them potential candidates for quantitation with real-time PCR. This led to real-time PCR studies of those genes that showed the largest change with the jump study. The results of these studies are presented below.

Additional Progress for Technical Objective 1

1) Rationale for our new strategy

We found that neither pQCT nor bone markers showed large enough differences required for QTL studies with respect to control in the jump-up and jump-down regimen. Hence, we elected to use microarray and real time PCR methods to analyze those genes that responded to mechanical loading. We evaluated 8,734 genes on the GEM I microarray chips with a threshold of light intensity of 1000 relative units in which 58% of the accessions in the chips were detected at a conservative threshold. On a more liberal threshold of 400 relative units, 88% of the accessions were detectable in the mouse femur of C57BL/6J strain. Out the 58%, 93 genes and ESTs had fluorescence signals higher than 10,000 relative units. Using a PUBMED search, we found that 31 out of 91 genes are expressed in the musculoskeletal system. These are shown in **Table 5.**

Table 5. List of genes expressed in musculoskeletal system with signals above 10,000 relative units in microarray study

Singal Level	Genes names	Expression/function
37,831	Procollagen, Type I, alpha	Bone formation
32,419	Lactotransferrin	Bone marrow
29,700	CD24a antigen	Bone marrow and liver
24,486	Hemoglobin alpha, adult chain	Fetal liver, spleen, bone marrow
22,792	Nuclear receptor binding factor 1	Bone
21,503	Glyceraldehyde-3-phosphate dehydrogenase	Bone
19,642	Carbonic anhydrase 2	Osteoclast

19,615	Tubulin alpha 4	Bone marrow stromal cells
19,485	Histocompatibility 2, L region	T cell
19,215	Interferon gamma receptor	Bone marrow
17,889	Histocompatibility 2, L region	Bone marrow
16,708	Secreted phosphoprotein 1	Bone
16,004	ESTs moderately similar to phosphatidylinositol	Osteoclast
15,396	S100 calcium binding protein A8	Bone /teeth
14,295	Interferon-dependent positive-actin transcription factor 3 gamma	Bone resorption
14,264	Hemoglobin alpha, adult chain 1	Bone marrow and fetal liver
12963	*Mus musculus* mRNA for Zinc finger protein s11-6, complete cds	Osteoblast
12,861	Lipocalin 2	Bone
12,419	CCAAT/enhancer binding protein (C/EBP), alpha	Osteoblast
12,376	Myeloperoxidase	Hematopioetic cells, bone marrow
12,070	*M.musculus* endothelial monocycte-activating polypeptide I mRNA, complete cds	Osteoblast
12,044	Beta-2-microglobulin	Bone
11,909	RAB11B, member of RAS oncogene family	Bone
11,356	Calponin 2	Muscle
11,311	Tubulin, beta 5	Bone marrow, spleen developing liver and lung
11,193	Myeloperoxidase	Myeliod cells
11,056	Enolase 3, beta muscle	Muscle
10,991	ATPase-like vaculor proton channel	Osteoclast
10,942	Cathelin-like protein	Host defense and wound repair
10,451	*M.musculus* myosin light chain 2mRNA, complete cds	Muscle
10.268	*M.musculus* Btk locus, alpha -D-galactosidase A (Ags), ribosomal protein (L44L) and Bruton's tyrosine kinase (Btk) genes, complete cds	B-cell

2) Isolation of RNA from pooled tibia and femur from B6 mice for Microarray analysis

Mechanical training was performed on the B6 mouse strain. RNA from the tibia and femur were pooled together from experimental (jump trained) and control mice and used for microarray to evaluate genes that showed maximal differences in expression between the two groups. The data was analyzed using Guantarray software. Interestingly, we found that Procollagen type 3 and osteocalcin showed high expression with jumping compared to the control. As 100μg of RNA was used for each sample, this method could not be applied to individual mice. Hence, we optimized the conditions for the microarray method to reduce the amount of RNA needed for microarray (see Technical Objective 3).

3) Development of a real time PCR method for quantification of candidate gene found by microarray

To quantify and compare the microarray results of the peak gene expression found in response to our mechanical loading studies, we used a technique called real time (RT) PCR. Reverse transcription followed by RT-polymerase chain reaction (PCR) is the

technique of choice for analyzing and quantifying the peak mRNA expression and also for comparing the results with microarray when RNA is in low abundance. We have designed and developed an assay with an optimal condition for obtaining accurate results using smart cycler real time PCR under a syber green dye method. The principle of this instrument is similar to an ordinary PCR with the exception being that it utilizes a fluorescent dye that binds to amplified product and emits fluorescent signals that are viewed in terms of cycles and are used for measuring the difference of expression between genes. After optimizing all the necessary conditions, oligonucleotide primers were designed according to target and the PCR reactions were performed in log concentration of a sample RNA. In addition, we also used an internal control B-actin to analyze the results. The syber green dye method showed a high specificity and sensitivity with high test linearity of Pearson correlation coefficient r>0.99. This method was used to analyze the results of the jumping regimen.

Table 6. Quantification of Type-I collagen (Col A1) in the bones of control vs. jumped mice using real time PCR.

	Cycles ± SD			
Groups	**Gene**	**1μg of RNA**	**0.1μg of RNA**	**0.01μg of RNA**
JUMP DOWN-20cm				
Control	Col A1	15.8±0.71	19.56±1.03	24.55±0.55
Jump-down 20 cm	Col A1	15.68±0.53	24.4±0.97	27.01±1.61
Control	β-actin	21.77±1.7	23.49±1.07	27.71±1.44
20cm Jump-down	β-actin	21.34±0.89	22.69±0.96	27.37±0.40
JUMP-DOWN-40cm				
Control	Col A1	18.62±4.03	19.83±0.72	23.58±1.11
Jump-down 40cm	Col A1	16.24±2.24	19.4±0.7	23.39±0.87
Control	β-actin	19.3±0.84	21.98±0.51	26.07±0.93
40cm Jump-down	β-actin	25.66±10.28	22.75±1.122	25.41±1.62
JUMP-UP 20cm				
Control	Col A1	24.4±3.05	28.32±2.06	28.82 ± 0.86
Jump-up 20cm	Col A1	24.25±2.55	28.65±1.22	28.82 ± 0.86
Control	β-actin	30.51±3.51	34.72±2.56	36.83± 0.43
Jump-up 20cm	β-actin	30.84±1.6	35.51±1.33	36.91 ± 1.04

P>.05 for all co-variance

4) Experimental Details

Comparing the expression of collagen (ColA1) in the Jump-up and Jump-down regimens by means of Real time PCR After optimizing the microarray conditions, we evaluated the expression of the ColA1 gene in response to mechanical stimuli. Six-week old mice of B6 strain alone were given mechanical training with different types of jumping, as shown in **Table 6**. The animals were trained for four days and were sacrificed after 24 hours. The RNA isolated from these animals, in terms of quantity and quality, was sufficient for our experiments. In this experiment, we used type-I collagen since it forms 95% of bone matrix and therefore an increased expression would be effected in response

to loading. For the statistical analysis, we had three sub-groups in each jumping regimen. The RNA was reverse transcribed using RT and cDNA was obtained. The real time PCR was performed using 1μg, 0.1μg and 0.01μg concentrations. Each reaction was performed with a positive control B-actin. In neither of the jumping regimens (height and times) were we able to find any significant changes on the Type-I collagen gene expression. The results are tabulated and shown in Table 6. This data indicates that the jumping regimens did not produce sufficient mechanical strain to be detected by our RT-PCR technique.

B. Technical Objective 2:

Our goal for this technical objective is to conduct a QTL analysis in F2 mice separated by sister-brother mating of C3H-B6F1 using the jump training technique to elicit the quantitative phenotype of periosteal bone formation. We have proposed to carry out Technical Objective 2 in Year 2.

C. Technical Objective 3:

Our goal is to evaluate the effects of mechanical signaling using a physiologically relevant CytoDyne flow chamber in order to produce a fluid flow shear strain for evaluation of proliferation and differentiation and also for studies of gene expression and signal transduction pathways in cultures of C3H and B6 mouse osteoblasts.

This report includes our progress for the first twelve months of our proposed work. The specific objectives for Technical Objective 3 as described in our proposal are:

1) Apply the CytoDyne flow chamber to evaluate mechanical signaling of osteoblast cells *in vitro*. This will first involve the development of the optimal flow-conditions to obtain measurable responses to two *in vitro* parameters, namely bone cell proliferation and differentiation. Differentiation will be assessed initially by alkaline phosphatase activity and, subsequently, will be confirmed by other differentiation markers, such as N-terminal procollagen peptide production for example.
2) Evaluate dose-response in terms of levels of strain and continuous vs. pulsated flow.
3) Determine the bone cells' time response to optimal flow conditions. The doses of fluid flow shear strain will range from 0.5 to 10 dynes/cm^2.
4) Phenotypic analysis will be made in 8-week old mice, whereas it is easier to culture osteoblasts from newborn mice. Therefore, we will compare the phenotypic differences between the C3H and the B6 mice in response to fluid flow shear strain to determine if these are evident in bone cells derived from newborn mice; in which case, we would use newborn mice as a surrogate for our 8-week old mice.
5) Once the optimal conditions for fluid flow shear strain have been established, we will begin to evaluate strain-induced tyrosine phosphorylation levels of key signaling proteins by immunoprecipitation-immunoblotting approach using available antibodies.

6) Once we have established the optimal fluid flow shear strain conditions, we will begin to apply microarray technology to evaluate gene expression in osteoblasts exposed to fluid flow shear strain.

We have accomplished most of the above specific objectives; our progress in each of these specific objectives is given below.

Progress on Technical Objective 3

Several *in vitro* models have been developed to evaluate mechanical signaling in the past. Application of mechanical force to bone can produce two different localized mechanical strains on bone cells: 1) deformation of extracellular matrix (physical strain); and 2) increased extracellular fluid flow (shear strain). Accordingly, there are at least two forms of mechanical strain that can be applied to cultured bone cells: physical strain that is created by physical deformation of the cell by application of mechanical stress on the bone matrix and fluid flow strain that is caused by the changes in blood flow and interstitial fluid flow due to loading induced intraosseous pressure changes.

Several reports in the literature have indicated that fluid flow shear strain would be more relevant to bone cell metabolism than the physical strain. In this regard, Dr. Frangos of the University of California at San Diego has developed a Cytodyne Flow Chamber that could generate a defined fluid flow shear strain on cultured cells, including osteoblasts. Consequently, we decided to set up the Cytodyne Flow Chamber in our laboratory for our investigation of fluid flow shear on cultured bone cells.

We elected to determine the optimal conditions for application of the CytoDyne flow chamber using human osteosarcoma cells for Specific Objectives 1, 2 and 3. These conditions were then used to make specific evaluations of the differential response of C3H and B6 mice in Specific Objectives 4, 5 and 6.

Specific Objective 1: Development of an in vitro system to apply defined mechanical strains to cultured bone cells. We used physiologically relevant flow shear strains of 20 dynes/cm^2 and measured the effect on [^{3}H] thymidine incorporation (an index of cell proliferation) and the specific activity of alkaline phosphatase (a marker of osteoblast differentiation) in normal human mandible-derived osteoblasts. We found that application of steady fluid flow shear strain of 20 dynes/cm^2 for 30 minutes on normal human osteoblasts caused a significant ($p<0.001$) increase (50-100%) in the [^{3}H] thymidine incorporation 24 hours later compared to control cells without the shear strain. The same shear strain also induced a significant ($p<0.001$) increase (25-75% compared to unstrained controls) in the specific activity of alkaline phosphatase assayed at 24 hours (**Figure 2**).

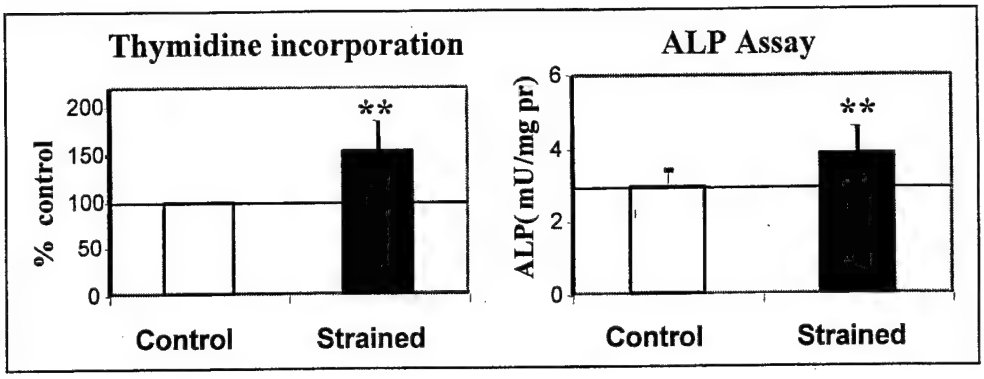


Figure 2. [3H]thymidine incorporation (left) and ALP specific activity (right) in human osteoblasts subjected to 30 minute steady fluid flow shear strain. Cells were subject to fluid flow of 20 dynes/cm^2 for 30min. [3H]thymidine incorporation and ALP specific activity were measured after 24hours . **p<0.001.

Specific Objective 2: Evaluate dose response in terms of levels of strain and continuous vs. pulsated flow. In order to study the effect of levels of shear strain, we used two different magnitudes of shear strain (i.e. 20dynes/cm^2 and 50 dynes /cm^2). As shown in **Figure 3**, [^{3}H] thymidine incorporation was significantly (p<0.001) increased in response to shear strain of 20 dynes/cm^2 as well as 50 dynes /cm^2 in human osteosarcoma TE85 cells *in vitro*. However, there was no significant difference in the [^{3}H] thymidine incorporation induction between the two levels of shear strain. These results indicate that higher levels of shear strain might be needed to induce bigger response in cell proliferation. Since fluid flow higher than 50 dynes /cm^2 would be beyond the physiological level of stress, we focused our studies at 20dynes/cm^2.

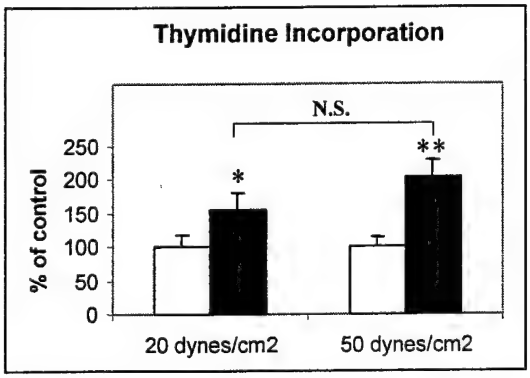


Figure 3 Dose response of [^{3}H] thymidine incorporation to shear strain. Cells were subjected to fluid flow of 20-50 dynes/cm^2 for 30min. [3H]thymidine incorporation was measured after 24 hours .*p<0.01,**p<0.001. Empty bars represent control cells while filled bars represent strained cells.

To study the effect of continuous vs. pulsatile fluid flow on the bone cell proliferation, normal human osteoblasts were subjected to a fluid flow shear strain of 20 dynes/cm^2 for 30 minutes continuosly or pulsatile flow (10 minute flow and 10 minute rest period with a total of 30 minute flow). The control cells were not subjected to any fluid flow. **Figure 4** shows that, as compared to the control cells, there was a significant

($p<0.001$) induction in cell proliferation in response to steady, (~50%) as well as pulsatile (~80%), fluid flow shear strain.

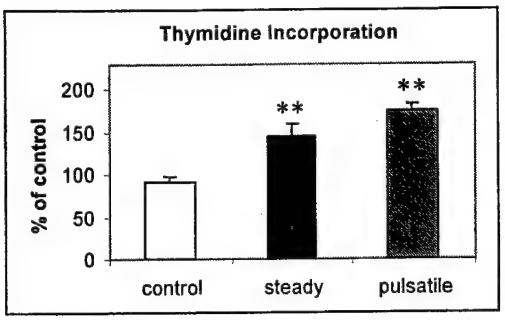


Figure 4. Effect of steady or pusatile fluid flow on [^{3}H] thymidine incorporation. Normal human osteoblasts were subjected to steady or pulsatile fluid flow of 20 dynes/cm^2 for 30 min. [3H]thymidine incorporation was measured after 24 hours. **$p<0.001$.

Specific Objective 3: Determine a time response of the bone cells to optimal flow conditions. Based on the studies above, we chose to use continuous fluid flow shear strain of 20 dynes /cm^2 as the optimal flow. To further determine the optimal time to obtain a measurable response in the cell proliferation, human osteosarcoma TE85 cells were subjected to steady flow of 20 dynes /cm^2 for different time points (30 minutes to 10 hours). As seen in **Figure 5**, there was a time-dependent significant increase in [^{3}H] thymidine incorporation for up to 4 hours as compared to the control cells not subjected to any flow. After that time point, cell proliferation induced by fluid flow starts to decrease with no significant induction at 10 hours. Based on these results, we decided to use steady fluid flow of 20 dynes/cm^2 for 30 minutes as the optimal conditions to get a response in cell proliferation as well differentiation.

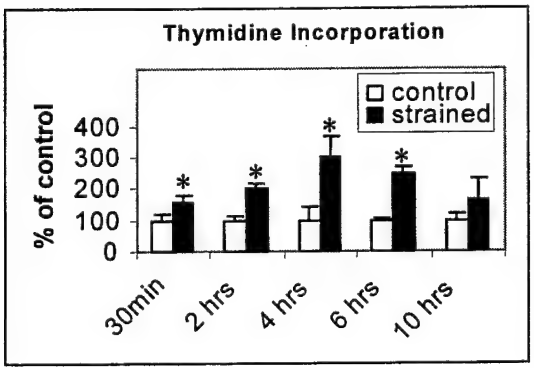


Figure 5. Time course response of [^{3}H] thymidine incorporation to shear strain. Cells were subjected to fluid flow of 20 dynes/cm^2 for 30min to 10 hours. [3H]thymidine incorporation was measured after 24 hours . *$p<0.01$.

Specific Objective 4: Compare the phenotypic differences between the C3H and B6 mice in response to fluid flow shear strain. Adult C3H mice have shown a 50% greater peak bone density compared to B6 mice. In addition, recent phenotypic studies comparing the skeletal response to mechanical loading and unloading *in vivo* between C3H and B6 mice revealed that C3H mice were relatively unresponsive to both mechanical loading and unloading as compared to B6 mice (1,2). This led us to postulate that the mechanical signaling mechanism in C3H bone cells is probably defective. To test this hypothesis, osteoblasts were isolated from six-week old C3H and B6 mice by sequential collagenase digestion and subjected to a fluid flow shear strain of 20

dynes/cm^2 for 30 minutes. Our data shows that there was a significant (P<0.001) increase in [^{3}H] thymidine incorporation to the shear strain in B6 osteoblasts whereas no such increase was noted in C3H osteoblasts (**Figure 6**). A similar difference on ALP specific activity in response to fluid flow shear strain was observed between bone cells derived from C3H mice and on those derived from B6 mice.

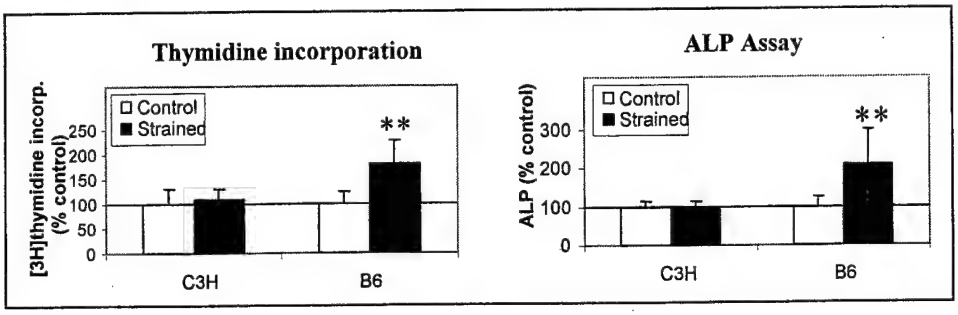


Figure 6. [3H]thymidine incorporation (left) and ALP specific activity (right) in osteoblasts from C3H and B6 mice subjected to 30 minute steady fluid flow shear strain. Cells were subject to fluid flow of 20 dynes/cm^2 for 30min. [3H]thymidine incorporation and ALP specific activity was measured after 24hours . **p<0.001.

Specific Objective 5: Evaluate strain-induced tyrosine phosphorylation levels of key signaling proteins in C3H and B6 mice. In order to clarify the signaling pathways that could possibly lead to the changes in fluid flow induced cell proliferation and differentiation in B6 bone cells and not in the bone cells isolated from C3H mice, we studied the fluid flow induced phosphorylation of MAPK and integrin expression in these cells. As shown in **Figure 7**, fluid flow did not have any significant effect on the integrin β1expression in the C3H bone cells. Interestingly, B6 bone cells showed a significant increase in the integrin β1 expression when subjected to fluid flow.

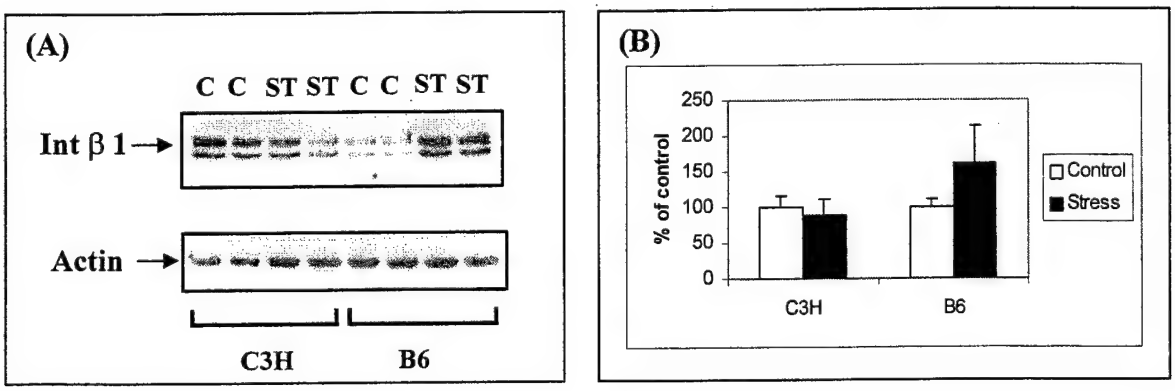


Figure 7. Effect of shear stress on integrin β1 in osteoblasts isolated from C3H and B6 mice. Cells were subjected to fluid flow of 20 dynes/cm2 for 30 minutes. **(A).** Cell lysates were immunoblotted with anti-integrin β1 and anti-actin antibodies. **(B).** The graph represents the densitometric measurements of integrin β1 levels from western blots normalized by actin. "C" represents control;"ST" represents strained.

Similarly, when the effect of fluid flow on MAPK phosphorylation was studied, it was observed that there was a significant increase in the phosphorylation levels of both the p44 and p42 in B6 bone cells when subjected to fluid flow. However, in C3H bone cells, no change was noted in response to fluid flow in either p44 or p42 phosphorylation levels (**Figure 8**).

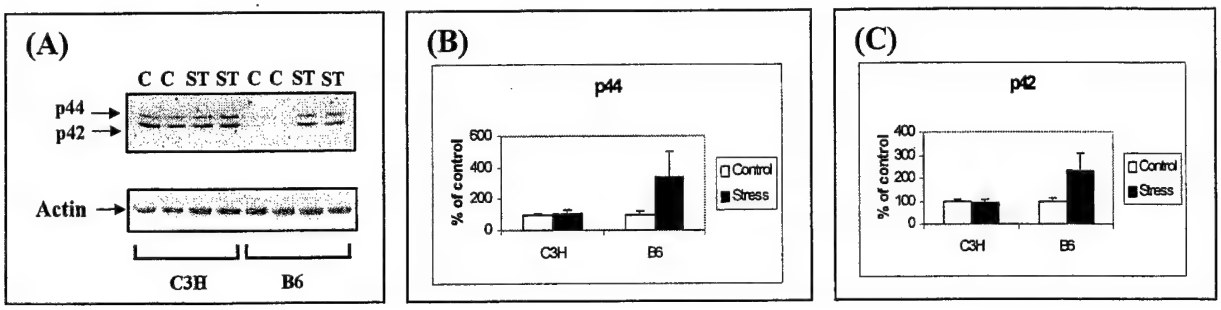


Figure 8. Effect of shear stress on phosphorylation levels of MAPK in osteoblasts isolated from C3H and B6 mice. Cells were subjected to fluid flow of 20 dynes/cm2 for 30 minutes. **(A):** Cell lysates were immunoblotted with anti-phospho-ERK and anti-actin antibodies. **(B&C):** The graph represents the densitometric measurements of p44 and p42 phosphorylation levels from western blots normalized by actin. "C" represents control;"ST" represents strained.

Specific Objective 6: Determine the optimal concentration of RNA for microarray technology to evaluate gene expression in C3H and B6 bone cells in response to fluid flow. In order to apply fluid flow shear strain to the bone cells using the CytoDyne flow chamber, cells are plated on glass slides (75x38mm) at 5x 10^4 cells/slide. Thus, the total amount of RNA extracted from a single slide is very low (2-3μg). Previous kits (Cyscribe) required at least 100μg of RNA for labeling procedure in order to analyze gene expression. In order to optimize for the smaller amount of RNA, we used recently developed Micromax TSA labeling and detection kit from PerkinElmer, as the supplier's recommendation was to use 1μg of RNA. To optimize the amount of RNA needed to do the microarray analysis, we used different concentrations of RNA (2μg-0.125μg). Total RNA was extracted from MRL mouse liver and gene expression was analyzed using in-house microarray slides containing ~6000 genes. **Figure 9** shows the expression levels of number of genes expressing low (0-1000), intermediate (1000-10000) and high (10000-70000) Cy3 signal values. As shown in the figure, there was no difference in number of genes between 1μg and 2μg of total RNA. At 0.5μg of RNA, more genes were expressed at lower intensity and a fewer number of genes were expressed at a higher intensity. At 0.125μg of RNA, the amount of RNA was too low, as most of the genes were expressed at low level of Cy3 signal value. Therefore, we decided to use 1-2μg of RNA for the future evaluation of gene expression in C3H and B6 bone cells in response to shear strain.

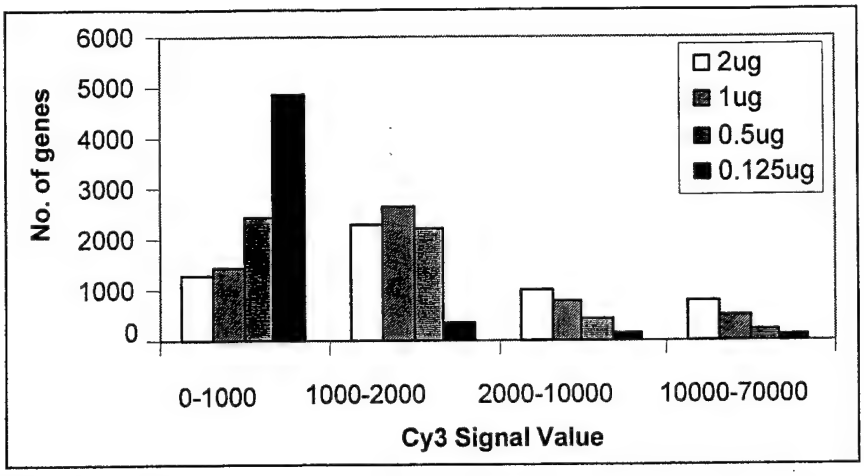


Figure 9. Number of genes expressing low (0-1000), intermediate (1000-10000) and high (10000-70000) Cy3 signal values.

Reportable Outcomes

Publication:

Umemura Y, Baylink DJ, Wergedal JE, Mohan S, and Srivastava AK: A time course of bone response to jump exercise in C57BL/6J mice. J Bone Miner Res 2002 (in press).

Conclusions - Molecular Genetics Studies on Bone Mechanical Strain

1) Microarray studies showed that with over 8,000 genes explored, 93 of these genes showed a more than 10 fold increase in expression above background. 36 of these genes were interpreted on the basis of findings in PUBMED to be associated with the musculoskeletal system. These 36 genes were then used in subsequent studies to further evaluate microarray differences between jump trained animals and controls.
2) We have optimized the conditions for Microarray technique.
3) We have evaluated the gene expression in response to loading using microarray to look for the candidate genes.
4) To quantify the Microarray results, we have set up a method using RT-PCR.
5) We found that RT-PCR results did not reveal any significant gene expression changes in jump-up or jump-down training.
6) All of the studies using RT-PCR indicate that the method is reproducible and that the method can be applied using small amounts of RNA, allowing the measurement of candidate gene expression in our sample preparation to be quantified in each animal. However, the changes were not statistically significant in response to jump training using the collagen (ColA1) gene expression as an end point. We still feel that using the RT-PCR gene expression as an end-point is suitable for our purposes. However, the work shows that we require a greater mechanical strain to illicit sufficient changes in order to be able to quantitatively phenotype each animal for the QTL analysis. Therefore, in the future, we will continue to evaluate candidate gene expression by microarray and RT-PCR as our end point but will switch to 4-point bending as our means to illicit mechanical strains rather than jump training.
7) We developed an *in vitro* system to apply defined mechanical strain to cultured bone cells.

8) We evaluated dose response in terms of levels of strain and continuous vs. pulsated flow.
9) We also determined a time response of the bone cells to optimal flow conditions.
10) We compared the phenotypic differences between the C3H and B6 mice in response to fluid flow shear strain and found that osteoblasts isolated from B6 mice were significantly responsive to shear strain in terms of cell proliferation and differentiation. On the other hand, osteoblasts isolated from C3H mice were unresponsive to the same shear stress.
11) We evaluated strain-induced tyrosine phosphorylation levels of key signaling proteins in C3H and B6 mice. We studied the fluid flow induced phosphorylation of MAPK and integrin expression in these cells and found that shear strain induced a significant increase in the integrin β1 expression as well as the phosphorylation levels of p44 and p42 in B6 bone cells. On the contrary, no change was observed in either integrin β1 expression or phosphorylation levels of p44 and p42 in C3H bone cells in response to fluid flow.
12) We determined the optimal concentration of RNA for microarray technology to evaluate gene expression in C3H and B6 bone cells in response to fluid flow.

References

1. Akhter MP, Cullen DM, Pedersen EA, Kimmel DB, Recker RR. *Bone response to in vivo mechanical loading in two breeds of mice.* Calcif Tissue Int 1998 Nov; 63(5):442-9
2. Kodama Y, Uemura Y, Nagasawa S, Beamer WG, Donahue LR, Rosen CR, Baylink DJ, Farley JR. *Exercise and mechanical loading increase periosteal bone formation and whole bone strength in C57BL/6J mice but not in C3H/Hej mice.* Calcif Tissue Int 2000 Apr;66(4):298-306

Appendices

Appendix 1-1:
Umemura Y, Baylink DJ, Wergedal JE, Mohan S, and Srivastava AK: A time course of bone response to jump exercise in C57BL/6J mice. J Bone Miner Res 2002 (in press).

2. Genetic Analysis of Three large Pedigrees with Very High Bone Density

Introduction

Peak bone mass, an important determinant of fracture risk in the elderly, is under genetic control. Thus, the identification of genes regulating peak bone density may represent a major advance in both the understanding of pathways that regulate bone density and the pathogenesis of bone diseases such as osteoporosis.

Recent studies demonstrate that carefully selected family-based study designs utilizing linkage analysis are generally more informative than association studies in identifying genes that regulate complex processes such as peak bone density. Our long-term goal in this is proposal is to identify those genes that contribute to peak bone density by linkage analysis studies in families with very high BMD (>2.5 SD).

Three large pedigrees with affected members exhibiting bone density of more than 2.5 SD above the young normal mean were collected. These pedigrees provide an excellent opportunity for studies on the linkage of genes responsible for high bone density and the evaluation of the clinical utility of identified candidate genes in the diagnosis and treatment of bone disorders.

A. Technical Objective 1: To measure BMD and biochemical markers of bone formation and resorption and collect clinical information on all family members belonging to the three pedigrees in order to confirm that the skeletal phenotype of affected members with high BMD in our three pedigrees is different from the phenotypes of known genetic diseases which are characterized by a high BMD. The specific objectives for the first twelve months, as outlined in our proposal, are:

1) All subjects belonging to the three selected pedigrees will have a history and physical examination and complete a questionnaire that provides information on lifestyle factors that might influence bone density.
2) All subjects belonging to the three pedigrees will have a bone density measurement of the spine and the hip.
3) All subjects belonging to the three pedigrees will have serum biochemical markers of bone formation and resorption (serum sALP, serum osteocalcin and serum N-procollagen peptide as bone formation markers and urine NTX, urine C-telopepetide, and serum TRAP 5B assays as bone resorption markers).
4) We will compare the results of the above evaluations with similar information on patients with known genetic causes of high bone density. This analysis, plus the genotype analysis described below, will assist us in choosing those pedigrees to focus upon for genotyping.

We have accomplished most of the above specific objectives. Our progress in each of the Specific Objectives is given below.

Progress on Technical Objective 1

Specific objective 1: Blood was collected for DNA and bone marker studies from 146 members. We collected medical and dietary histories and performed physical examinations on all subjects belonging to the three selected pedigrees.

Specific objective 2: Bone mineral density (BMD) was measured in approximately 146 members from three families known for high BMD (47 BMDs Family Z, 46 BMD Family E, 53 BMD Family X). From those family members evaluated for BMD, a family history and a dietary record were obtained and blood was collected. At present, we have identified 19 family members with extremely high BMD (>2.5 SD) (9 members in Family E, 3 members in Family X, 7 members in Family Z) and one member with very low BMD (Family Z).

Specific objective 3: 146 obtained serum samples were analysed using bone turnover markers with standard techniques. Total serum alkaline phosphatase, as well as PINP, were measured and adjusted for age.

We have performed bone marker analysis between subjects with high bone density and age-matched normal members. The data for analysis performed on family E is given in the following two tables and figure.

Table 7. Bone density and bone markers from subjects with high bone density and age-matched normal control in the E family*.

Sample	Age	T-hip	T-spine	Z-hip	Z-spine	PINP	SALP	HALP	TALP
57	20	-1.48	-1.24	-0.42	-0.26	64.9	2.5	11.2	13.6
66	22	**9.34**	**6.58**	**8.93**	**6.43**	**56.9**	**5.8**	**12.1**	**17.9**
73	22	**11.31**	**8.67**	**11.26**	**8.82**	**110.7**	**5.5**	**21.0**	**26.5**
77	23	2.27	1.59	2.27	1.39	46.1	8.3	19.3	27.7
65	28	**14.47**	**8.44**	**12.13**	**8.73**	**45.1**	**4.0**	**12.5**	**16.5**
82	29	-0.27	0.74	-0.05	0.72	67.8	3.2	19.0	22.1
74	30	**12.01**	**8.75**	**11.69**	**9.48**	**65.4**	**2.8**	**20.8**	**23.7**
71	31	0.01	1.16	-0.29	0.82	31.3	2.4	16.2	18.6
64	32	**11.22**	**9.33**	**10.93**	**10.87**	**28.4**	**1.2**	**16.0**	**17.2**
60	34	-0.29	0.22	0.03	0.19	59.6	4.3	20.5	24.8
31	50	0.76	1.72	2.65	2.03	40.0	9.1	17.4	26.5
22	52	**9.71**	**9.38**	**9.37**	**10.36**	**69.7**	**3.3**	**26.8**	**30.1**
17	58	**5.6**	**8.9**	**9.33**	**8.7**	**40.9**	**9.4**	**23.4**	**32.9**
41	57	-0.86	-0.86	0.17	0.11	25.5	3.1	18.3	21.3
HBD	Av	10.52	8.58	10.52	9.06	59.59	4.57	18.94	23.54
HBD	SD	2.54	2.92	3.25	2.96	54.56	5.17	19.67	24.84
Normal	Av	0.02	0.48	0.62	0.71	47.89	4.70	17.41	22.09
Normal	SD	1.21	1.16	1.27	0.79	16.66	2.81	3.07	4.88

"T-hip" = hip BMD T-score; "T-spine" = Spine BMD T-score; "Z-hip" = Hip BMD Z-score; ""Z-spine" = Spine BMD Z-score. * Data from high bone density subjects are highlighted in bold font.

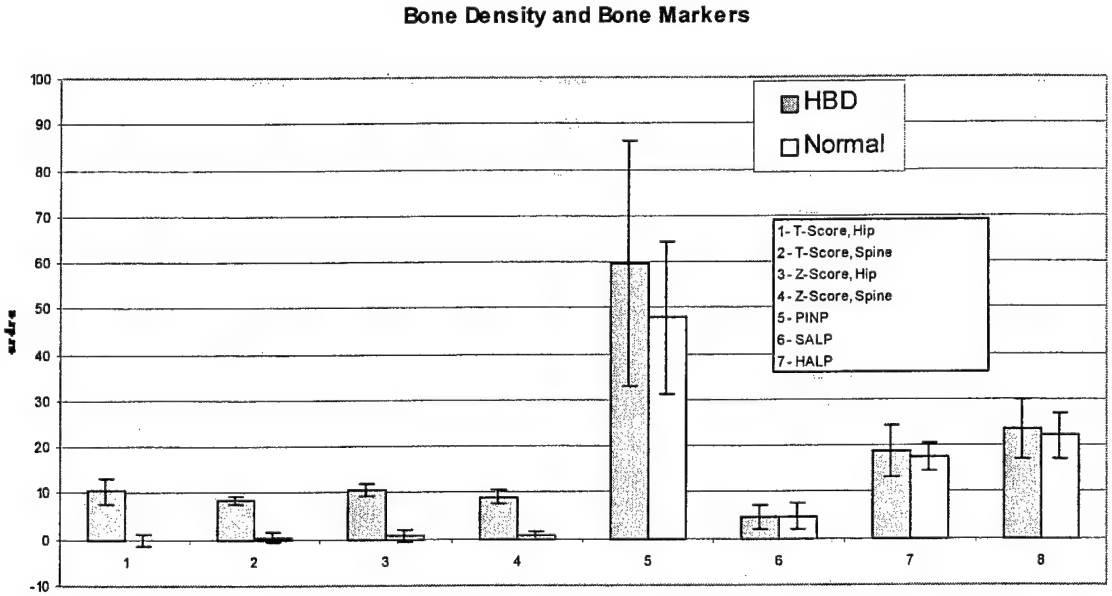
Table 8. Reference ranges for bone markers.

Test	Abbrev.	Reference Range	Units
Alkaline Phosphatase, skeletal	sALP	3.1 - 16.8	U/L
Alkaline Phosphatase, hepatic	hALP	8.6 - 32.2	U/L
Alkaline Phosphatase, total	tALP	14.4 - 45.8	U/L
Procollagen I N-terminal peptide, intact	PINP	19 - 84	ug/L

Analysis of serum bone marker results: In this study, we evaluated the N- terminal procollagen peptide assay (PINP) along with the skeletal alkaline phosphatase and the total alkaline phosphatase assays (**Figure 10**). These are all bone formation assays. In this preliminary analysis, we evaluated seven patients from the E family along with

corresponding age and sex match controls. The values for PINP were slightly higher in the E family high bone density subjects than the controls. This also applied to the total alkaline phosphatase assay. However, these changes were not statistically significant. The increase in the PINP in the E family high bone density patients was approximately 10% and it is conceivable that when further subjects are analyzed this change could become significant. In any case, the fact that we did not find a decrease in either procollagen peptide or alkaline phosphatase assays indicates that these patients do not have osteopetrosis, which is a disease of impaired bone resorption. Bone formation markers are typically decreased in osteopetrosis. Consequently, these data strongly suggest that the cause of the high bone density in the E family is not due to decreased bone resorption but instead due to an increase in bone formation. If so, the E family would constitute a family with osteosclerosis, which is a consequence of increased bone formation. Our original interest in the E family was because clinically these patients did not exhibit the complications that can accrue in osteopetrotic syndromes. Therefore, these assays are entirely consistent with the clinical observations that our high-density subjects in the E family have increased bone formation. This is a key observation because in the future, if these observations were confirmed with a large number of patients, we would then focus on genes known to enhance bone formation. Furthermore, this means that we can use osteoblasts, which are the bone forming cells, to evaluate candidate genes *in vitro* assays.

Figure 10. Bone density and bone markers from subjects with high bone density and age-match normal control in the E family



Specific objective 4: We have compared the results of the above evaluations with similar kinds of information on patients with known genetic causes of high bone density. This analysis, plus the genotype analysis described below, indicates that the high bone density in the E family is associated with high bone formation and not due to decreased bone resorption.

B. Technical Objective 2

After we collect DNA, we propose to begin targeted genotyping for high bone mass (HBM) locus and osteopetrosis locus. Our ultimate objective is to perform a genome-wide scan with well-spaced microsatellite markers in all three pedigrees in order to confirm that the chromosomal locations of candidate genes in the three pedigrees are: a) different from previously described genetic loci linked to BMD; and b) different from each other. This report includes our progress for the last twelve months of our proposal. The specific objectives for the first twelve months, as outlined in our proposal, are:

1) We will extract DNA for members of pedigrees E, X and Z as blood samples are collected from each family group.
2) As DNA becomes available, we will identify pedigree segments in each family (essentially nuclear family units) that are informative for linkage studies and inferences of regional haplotypes.
3) In the informative family segments, we will perform targeted genotyping for 5-10 SNPymorphic markers closely linked to the sites of genes previously known to cause increased BMD. We will use microsatellite markers and alleles will be assayed by standard PCR and electrophoretic techniques.
4) Genetic and phenotypic data from each family will be examined to determine if there is evidence for "haplotypic-sharing" in affected individuals and/or other evidence supporting genetic linkage of the BMD phenotype to any of the targeted sites.

We have accomplished most of the Specific Objectives above. Our progress in each Specific Objective is provided below.

Progress on Technical Objective 2

Specific Objective 1: DNA from 146 family members was extracted using DNA purification kits from Qiagen. The isolated DNA was dissolved in the 1 x TE-buffer for better stabilisation and frozen at -20^{0} in 2-3 aliquots.

Specific Objective 2: All information, including dietary records, BMD results, age, height, weight and serum markers, was organized in a special computer program (Progeny). Using this program, pedigrees for all five families were drawn. Twenty-two members from family E, including 8 individuals with high bone density, nineteen members from family X, including 3 individuals with high bone density, and Nineteen members from family Z, including 4 individuals with high bone density, were selected for linkage studies and inferences of regional haplotypes.

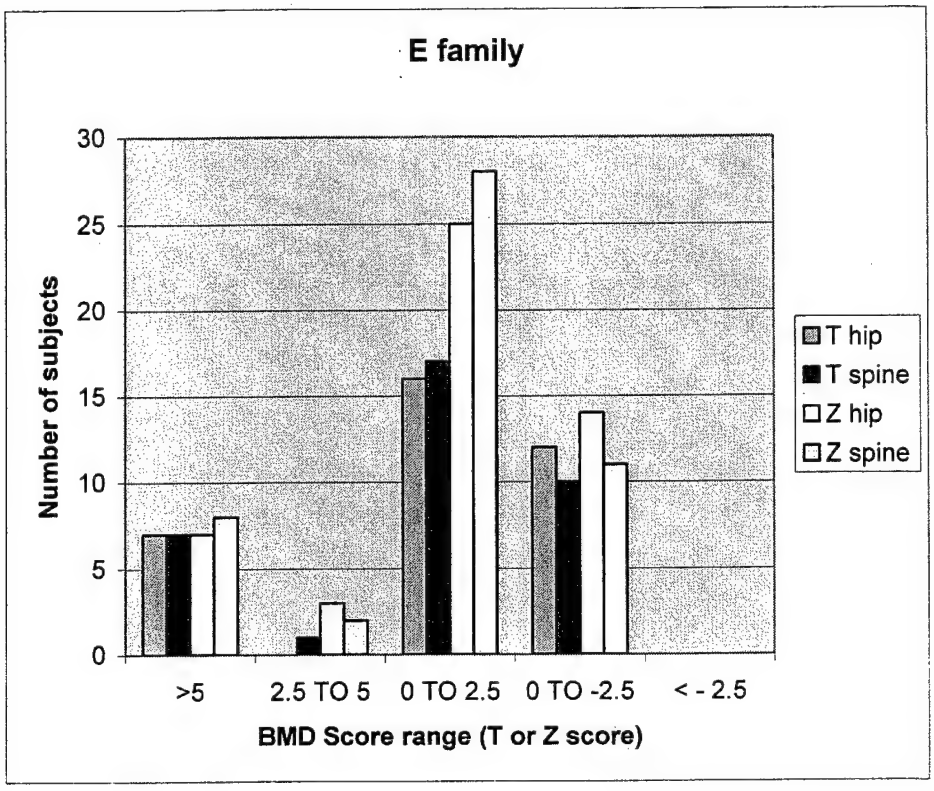


Figure 11. The distribution of bone density data for the E family.

Specific Objective 3: We began genetic testing using microsatellite markers for regions on chromosomes 1 and 11, where known peak bone density genes are located, to examine any linkage to those known genetic loci of high bone density. Altogether, 14 microsatellite markers on chromosome 11 (11q12-13) and 7 markers on chromosome 1 (1q21-23) were tested using PCR and gel electrophoresis techniques. Linkage analysis using a special genetic statistic program (GAP) is currently being performed.

Genetic genotyping analysis revealed that, in our three pedigrees, only family E is linked to chromosome 11. Families Z and X may represent an opportunity to identify new loci associated with high bone density.

Table 9. List of microsatellite markers used to genotyping the families

Marker	CM	Allelle size
D11S2630	33.1	90-106
D11S911	79.4	134-156
D11S914	37.6	274
D11S1968	43.5	174
D11S1784	63.1	145
D11S4083	47.0	200
D11S916	76.13	135-153
D11S935	45.9	196-208
D11S987	82.6	102-124
D11S1313	74.6	184-204
D11S1314	87.7	94-100
D11S1319	74.6	186-194
D11S1395	90.2	220-232
D11S4186	97.6	156-178
D1S218	196.5	266-286
D1S398	201.5	175
D1S426	202.1	141
D1S484	202.3	138
D1S498	165.3	197
D1S303	165.7	191
D1S2771	172.8	257
D1S2630	183.3	64

Specific Objective 4: We performed a study of core haplotype-sharing between the families and, specifically, between the subjects with high bone density. We failed to identify any haplotype-sharing in families Z and X. However, we identified haplotype sharing between seven subjects with high bone density in family E (see **Table 10**), the core haplotype for microsatellite markers of D11S935-D11S4083-D11S1319-D11S1395-D11S987-D11S1313-D11S916 is 1-3-1-2-1-1-1. This region of chromosome 11, from 45-76 cM, contained one locus for high bone density. Therefore, we undertook DNA sequencing of several candidate genes located in this chromosome region using four samples from family E (see Additional Progress below). As our results for the DNA sequencing of five candidate genes show, the mutant gene leading to high bone density in the E family must be different from previously discovered genes known to be responsible for high bone density.

Table 10. Core haplotype in the family E *

Markers	cM	**E17**	E60	**E65**	**E66**	**E64**	E102	E69	E72	E71	E104	E105	E106	E107	**E22**	**E73**	**E74**	E24	E75	E76	E77
D11S935	45	**1.1**	1.2	**1.2**	**1.2**	**1.2**	1.3	1.2	1.2	1.1	1.3	1.3	1.2	1.3	**1.1**	**1.3**	**1.4**	1.1	1	1	1.1
D11S4083	47	**1.3**	1.4	**3.2**	**3.2**	**1.3**	3.2	1.3	1.3	1.4	3.2	1.3	1.4	3.2	**3.2**	**3.4**	**3.4**	1.3	1	1	1.2
D11S1319	58	**1.1**	1.1	**1.1**	**1.1**	**1.1**	1.2	1.3	1.3	1.1	1.1	1.1	1.1	1.1	**1.1**	**1.3**	**1.3**	1.1	1	1	1.1
D11S1395	58	**1.2**	1.2	**2.2**	**2.2**	**2.2**	2.2	2.2	3.2	2.2	2.2	2.2	2.2	2.2	**3.2**	**1.2**	**3.2**	1.2	3	2	1.2
D11S987	67	**1.2**	1.2	**1.1**	**1.1**	**1.3**	1.3	1.5	1.5	2.5	5.5	1.5	1.5	5.5	**1.3**	**1.1**	**1.1**	1.2	2	1	1.6
D11S1314	73	**1.1**	1.1	**1.1**	**1.1**	**1.1**	1.2	1.2	1.2	1.2	2.3		2.3	2.3	**1.1**	**1.1**	**1.1**	1.1	1	1	1.4
D11S916	76	**1,2**	1,2	**1,1**	**1,1**	**1,1**	1.1	1.3	1.3	3.3	3.3	3.3	1.3	3.3	**1.2**	**1.1**	**1.1**	1.3	1	1	1.3
D11S4184	84	**1.2**	1.2	**1.2**	**1.2**	**1.2**	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	**1.2**	**1.2**	**1.2**	1.2	1	1	1.2

Haplotype is shown for different markers. The numbers represent different polymorphic alleles for the 2 chromosomes. *: The data from subjects with extremely high bone density is indicated in bold.

C. Additional Progress

In addition to accomplishing all the Specific Objectives in our original proposal, we have also performed DNA sequencing of five candidate genes located in the QTL of Chromosome 11. Initially, we have screened the polymorphism of five genes (LRP5, KCNE, LTBP3, KCNK4 and RAB30) on human chromosome 11 using 4 members of family E. The results are given below.

1. DNA sequencing of LRP5

Rationale

The low-density lipoprotein receptor–related protein (LRP)-5 functions as a Wnt co-receptor. Mice with a targeted disruption of LRP5 develop a low bone mass phenotype. Gong, et al (1) reported that mutations in LRP5 cause the autosomal recessive disorder *osteoporosis-pseudoglioma syndrome* (OPPG) and that OPPG carriers have reduced bone mass when compared to age- and gender-matched controls. Interestingly, Little, et al. (2) found that a special mutation in LRP5 confers results in the autosomal dominant-high-bone-mass trait. Therefore, different mutations in LRP5 can result in both low and high bone mass. We have observed that there are high bone density carriers and some low bone density carriers in the large E pedigree and that this pedigree also shows linkage to chromosome 11q12-13, where LRP5 is located. These observations make LRP5 a good candidate gene to carry out DNA sequencing.

Results

We have designed primers to cover all 23 exons of LRP5 and have performed DNA sequencing with four samples from the E pedigree (two with high bone density and two

with normal bone density). We observed some DNA sequencing changes in some of the exons. We are in the process of confirming the results via repeat DNA sequencing in both the same samples and with more high bone density samples from the E family. A summary of the sequencing results for LPR5 is provided in **Table 11**.

Table 11. DNA sequencing results for LRP5 *

Exon	E17(HBD)		E19		E22(HBD)		E24	
	Seq	SNP	Seq	SNP	SEQ	SNP	Seq	SNP
1	Y	A	Y	A	Y	B	Y	B
2	Y	A	Y	A	Y	A	Y	A
3	Y	N	Y	N	Y	N	Y	N
4								
5	Y	N	Y	N	Y	N	Y	N
6	Y	N	Y	N	Y	N	Y	N
7	Y	N	Y	N	Y	N	Y	N
8	Y	N	Y	N	Y	N	Y	N
9	Y	N	Y	N	Y	N	Y	N
10	Y	N	Y	N	Y	N		
11	Y	N	Y	N			Y	
12	Y	N	Y	N	Y	N	Y	N
13	Y	N	Y	N			Y	N
14	Y		Y				Y	
15	Y	N	Y	A	Y	N	Y	B
16	Y	N	Y	N	Y	N	Y	N
17	Y	N	Y	N	Y	N	Y	N
18	Y	N	Y	N	Y	N	Y	N
19	Y	N	Y	N	Y	N	Y	N
20	Y	N	Y	N	Y	N	Y	N
21	Y	N	Y	N	Y	N	Y	N
22	Y	N	Y	N	Y	N	Y	N
23	Y		Y	N			Y	N

*:"Y" = Yes and that we have performed DNA sequencing for the specific exon or sample. "N" = No DNA sequencing variants was found for that exon or sample. "A" and "B" represent two different polymorphisms identified in the same exon.

2. DNA sequencing of LTBP3

Rationale

The latent TGF binding protein 3 (LTBP3) has several functions with regards to TGF and bone formation. LTBP3 appears to: 1) target TGF to the matrix; 2) be capable of being proteolytically cleaved in order to release the latent TGF complex from the matrix; and 3) be involved in the activation of this form of latent TGF released from the matrix by cells. An independent function of this protein appears to be to serve as an extracellular matrix protein that forms large fibrillar structures which appear to be necessary for new bone

formation. Dabovic, et al (3) reported that decreased bone formation and bone abnormalities in LTBP3–null mice indicate a role for LTBP3 in modulating TGF-ß bioavailability. Furthermore, LTBP3 is located on chromosome 11q12-13 and is close to LRP5. For these reasons, we regard LTBP3 as a potential candidate gene for our high bone density pedigree E.

Results

There is no published data about the genomic structure of LTBP3, so we do not know how many exons and introns are present in LTBP3. To deduce the genomic structure of human LTBP3 (**Figure 12**), we obtained homologous genomic sequences to exons 1-2, 4-14, 19-22 and 27 by querying the NCBI high-throughput genome sequence (HTGS) database using BLAST. The intron size and DNA sequence of the intron-exon boundaries were determined by amplifying and sequencing PCR products using oligonucleotide primers designed to amplify across neighboring exons. By employing this method, we were able to design primers for exon 17 and exon 18 of LTBP3.

By comparing the cDNA and genomic DNA sequencing data from both human and mouse models, we inferred that LTBP3 has approximately 27 exons. We constructed the genomic structure of LTBP3 using software Vector NTI with DNA sequencing data from several BAC (**Figure 12**).

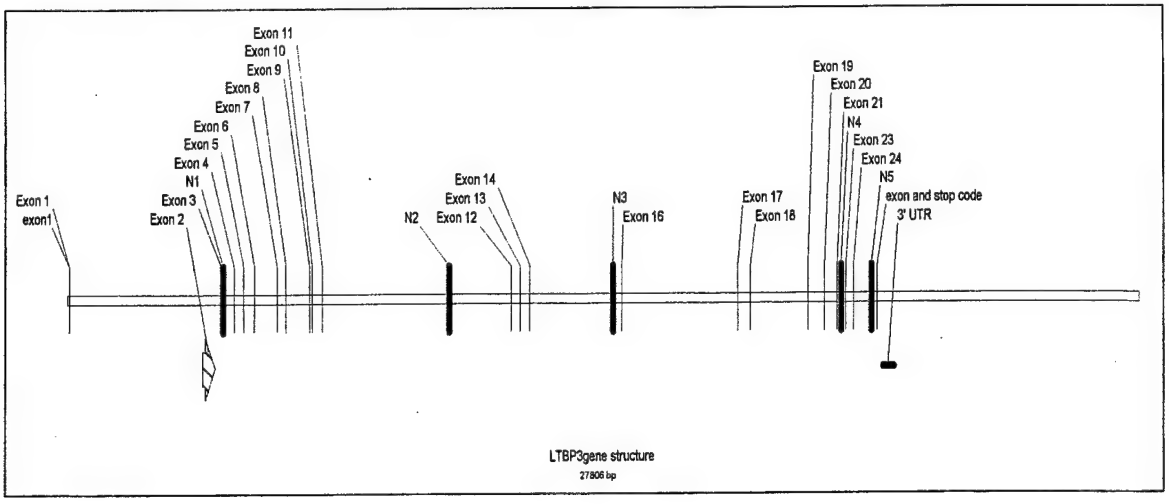


Figure 12. Proposed genomic structure of LTBP3

As shown in Figure 12, there are several gaps between the genomic sequencing of LTBP3, designated as N1 to N5. There is no DNA sequencing data available so far for the intron-exon boundaries for exon3, exon15, and exons 23-26. The results for DNA sequencing of LTBP3 gene are given in **Table 13**. No SNP was identified in four samples from family E in any of exons sequenced.

Table 13. DNA sequencing results for LTBP3

Exon	E17(HBD)		E19		E22(HBD)		E24	
	Seq	SNP	Seq	SNP	Seq	SNP	Seq	SNP
Exon1								
Exon2	Y	N	Y	N	Y	N	Y	N
Exon3								
Exon4	Y	N	Y	N	Y	N	Y	N
Exon5	Y	N	Y	N	Y	N	Y	N
Exon6	Y	N	Y	N	Y	N	Y	N
Exon7	Y	N	Y	N	Y	N	Y	N
Exon8	Y	N	Y	N	Y	N	Y	N
Exon9	Y	N	Y	N	Y	N	Y	N
Exon10	Y	N	Y	N	Y	N	Y	N
Exon11	Y	N	Y	Y	Y	N	Y	N
Exon12	Y	N	Y	N	Y	N	Y	N
Exon13	Y	N	Y	N	Y	N	Y	N
Exon14	T	N	Y	N	Y	N	Y	N
Exon15								
Exon16	Y	N	Y	N	Y	N	Y	N
Exon17								
Exon18								
Exon19	Y	N	Y	N	Y	N	Y	N
Exon20	Y	N	Y	N	Y	N	Y	N
Exon21	Y	N	Y	N	Y	N	Y	N
Exon22	Y	N	Y	N	Y	N	Y	N
Exon23								
Exon24								
Exon25								
Exon26								
Exon27	Y	N	Y	N	Y	N	Y	N

*:"Y" = Yes and that we have performed DNA sequencing for the specific exon or sample. "N" = No DNA sequencing variants was found for that exon or sample.

3. DNA sequencing of KCNK4

Table 14. DNA sequencing results for KCNK4

Exon	E17(HBD)		E19		E22(HBD)		E24	
	Seq	SNP	Seq	SNP	Seq	SNP	Seq	SNP
Kcnk4-1			Y					
Kcnk4-2	Y	A	Y	A	Y	A	Y	B
Kcnk4-3	Y	A	Y	B	Y	A	Y	A
Kcnk4-4	Y	N	Y	N	Y	N	Y	N

Kcnk4-5	Y	N	Y	N	Y	N	Y	N
Kcnk4-6	Y	N	Y	N	Y	N	Y	N

*:"Y" = Yes and that we have performed DNA sequencing for the specific exon or sample. "N" = No DNA sequencing variants was found for that exon or sample. "A" and "B" represent two different polymorphisms identified in the same exon.

4. DNA sequencing of KCNE

Table 15. DNA sequencing results for KCNE

Exon	E17(HBD)		E19		E22(HBD)		E24	
	Seq	SNP	Seq	SNP	Seq	SNP	Seq	SNP
Kcne	Y	N	Y	N	Y	N	N	N

*:"Y" = Yes and that we have performed DNA sequencing for the specific exon or sample. "N" = No DNA sequencing variants was found for that exon or sample.

5. DNA sequencing of RAB30

Table 16. DNA sequencing results for RAB30

Exon	E17(HBD)		E19		E22(HBD)		E24	
	Seq	SNP	Seq	SNP	Seq	SNP	Seq	SNP
RAB30-1	Y	N	Y	N	Y	N	Y	N
RAB30-2			Y	A	Y	B		
RAB30-3	Y	N	Y	N	Y	N	Y	N
RAB30-4	Y	A	Y	B			Y	B

*:"Y" = Yes and that we have performed DNA sequencing for the specific exon or sample. "N" = No DNA sequencing variants was found for that exon or sample. "A" and "B" represent two different polymorphisms identified in the same exon.

Reportable Outcomes

None

Conclusions - Genetic Analysis of Three large Pedigrees with Very High Bone Density

1) We collected physical examination information and dietary histories on 146 subjects from three large pedigrees with high bone density
2) We collected 146 blood samples for DNA extraction and bone marker assays. We extracted DNA from 146 samples and performed bone marker assays in 146 samples.

3) We measured bone density for 146 subjects, identifying 19 members with extremely high BMD (>2.5 SD).
4) The bone serum marker data showed no difference in bone formation in our high bone density E family compared to age and sex matched controls. We conclude that because formation is not depressed in these high bone density patients, as would be expected if these patients had osteopetrosis, the high BMD in these subjects is the result of an increase in bone formation. This observation is consistent with the clinical findings in these subjects. If this observation is confirmed with a larger number of subjects, we will then be able to focus on bone forming genes and utilize osteoblasts cultures *in vitro* to test candidate genes. According, the bone biochemical markers were a very critical aspect of our phenotypic characterization of the subjects with high bone density.
5) We carried out genotyping with 14 markers from chromosome 11 and 7 markers from chromosome 1. These two loci were previous reported as being linked to high bone density. In our three pedigrees, we found only family E to be linked to chromosome 11. DNA sequencing analysis results suggest that this could be a new high bone density gene. In addition, families Z and X may represent an opportunity to identify new loci associated with high bone density.
6) We performed DNA sequencing of five candidate genes. So far, there is no mutation or SNP in the subjects with high bone density compared with normal subjects. Therefore, we believe that the gene responsible for high bone density in family E is different with previously reported genes leading to bone disease associated with abnormal high bone density.
7) According to our results, our next step will be to perform a whole genome scan with two large pedigrees (the Z family and X family). In addition, we will continue our candidate gene studies with the family E.

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Appendices

None

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A time course of bone response to jump exercise in C57BL/6J mice

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Abstract Exercise, by way of mechanical loading, provides a physiological stimulus to which bone tissue adapts by increased bone formation. The mechanical stimulus due to physical activity depends on both the magnitude and the duration of the exercise. Earlier studies have demonstrated that jump training for 4 weeks produces a significant bone formation response in C57BL/6J mice. An early time point with significant increase in bone formation response would be helpful in: (1) designing genetic quantitative trait loci (QTL) studies to investigate genes regulating the bone adaptive response to mechanical stimulus; and (2) mechanistic studies to investigate early stimulus to bone tissue. Consequently, we investigated the bone structural response after 2, 3, and 4 weeks of exercise with a loading cycle of ten jumps a day. We used biochemical markers and peripheral quantitative computed tomography (pQCT) of excised femur to measure bone density, bone mineral content (BMC), and area. Four-week-old mice were separated into control ($n = 6$) and jump groups ($n = 6$), and the latter groups of mice were subjected to jump exercise of 2-week, 3-week, and 4-week duration. Data (pQCT) from a mid-diaphyseal slice were used to compare bone formation parameters between exercise and control groups, and between different time points. There was no statistically significant change in bone response after 2 weeks of jump exercise as compared with the age-matched controls. After 3 weeks of jump exercise, the periosteal circumference, which is the most efficient means of measuring adaptation to exercise, was increased by 3% ($P < 0.05$), and total and cortical area were increased by 6% ($P < 0.05$) and 11% ($P < 0.01$), respectively. Total bone mineral density (BMD) increased by 11% ($P < 0.01$). The biggest changes were observed in cortical and total BMC, with the increase in total BMC being 12% ($P < 0.01$). Interestingly, the increase in BMC was observed throughout the length of the femur and was not confined to the mid-diaphysis. Consistent with earlier studies, mid-femur bone mass and area remained significantly elevated in the 4-week exercise group when compared with the control group of mice. The levels of the biochemical markers osteocalcin, skeletal alkaline phosphatase, and C-telopeptide were not significantly different between the exercise and control groups, indicating the absence of any systemic response due to the exercise. We conclude that a shorter exercise regimen, of 3 weeks, induced a bone response that was greater than or equal to that of 4 weeks of jump exercise reported earlier.

Key words ■■

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Introduction

Physical activity or mechanical loading plays an important role in determining peak bone density. Physical activity creates loads on bone that have the potential to initiate bone formation and increase bone density [1–7]. Conversely, lack of mechanical loads results in rapid bone loss, as seen in immobilization studies [8–10]. A minimum effective strain (MES) [11–14], which is above average daily levels, is required to generate signals that are communicated to cells in bone tissue for adaptive bone formation through modeling. Physical activity levels above this MES can stimulate adaptive bone formation. Animal studies of bone adaptation have shown that mechanical regulation of bone due to physical activity is dependent on a combination of factors, including: (1) strain distribution; (2) strain magnitude; (3) number of repetitions; and (4) frequency [11–14].

We have previously reported [1] that mechanical loading, in the form of jump exercise, increases periosteal bone formation and bone strength in C57BL/6J mice, but not in C3H/HeJ mice. We used jump training as a model of exercise instead of treadmill exercise because jump exercise was found to be more effective in increasing limb bone mass in rodents [15]. In a previous study, 20 jumps/day for 4 weeks, which produced a bending strain of 176 N/mm^2 [1], was effective in gener-

ating the signal for adaptive bone response through modeling. The main aim of this study was to explore changes in femoral structural parameters after 2, 3, and 4 weeks of jump exercise. Data on the earliest bone response could provide useful information for mechanistic studies at the cellular level to investigate the initial stimulus to which bone tissue adapts by increased bone formation. In addition, an earlier time point than 4-week loading, if such elicited a significant bone formation response, would save time and effort in our genetic quantitative trait loci (QTL) studies to investigate genes regulating the bone adaptive response to mechanical stimulus. The secondary objective of this study was to evaluate the response to jump exercise by: (1) using peripheral quantitative computed tomography (pQCT) to measure changes in bone density, mass, and area; and (2) using biochemical markers to study systemic changes in bone metabolism. The pQCT would offer a less cumbersome and more precise tool to study bone response to loading as compared with bone histomorphometric measurements, which were used in an earlier study (1).

Materials and methods

Animals and treatment

All animal protocols used in this study had prior approval of the Animal Subjects Committee at the Chukyo University Graduate School of Health and Sport Sciences.

Four-week old C57BL/6J mice were obtained from Japan SLC (Hamamatsu, Japan) and acclimatized for 1 week on a 12-h light/dark cycle, with food (standard chow) and water available ad libitum. Mice were randomly assigned to either the jump-exercise or the non-exercised control groups (n = 6–7 mice/group). Details of the jump-exercise protocol have been described in the previous report [1]. In brief, each mouse in the jump groups was placed at the bottom of a special cage, 10 cm wide, 10 cm deep, and 25 cm high. The jumping exercise was initiated by applying an electrical current (80 volts) to the wire floor of the cage. Each mouse was housed individually and jumped from the floor of the cage to catch the top edge of the cage with its forepaws. The mouse was returned to the floor of the cage to repeat the procedure. The electrical current we used had an automatic turn-on phase and turn-off phase. We placed the mice on the stimulus plate at the turn-off phase and, after the first 3 days, most of the mice could jump before the turn-on phase. Each mouse in the three jump groups jumped ten times per day, 5 days a week, for 2, 3, and 4 weeks. The control mice were largely unstressed.

At the end of the experiment, blood was collected and femurs were dissected from both hind limbs. Serum was separated and kept at −70°C until analyzed for biochemical markers. Femurs were kept moist and frozen at −70°C. Frozen serum and femurs were analyzed at the J.L. Pettis Memorial Veterans Medical Center for biochemical analysis and bone density measurements, using peripheral quantitative tomography (pQCT).

Peripheral quantitative computed tomography (pQCT)

Bone density, mass, and area data were obtained from excised femurs (n = 6), and values were expressed as means ± SD. Two different thresholds were used to analyze the pQCT scans. The high threshold analysis gives the most accurate area results. The low threshold gives the most accurate mineral content analysis. The bones were scanned nine times, covering the entire length of the femur with each slice separated by 11% of the femur length. Slice 5, which is the mid-shaft slice, was used for all calculations and comparisons. Data from individual slices were used to plot total and cortical bone mineral content (BMC) (Fig. 1). Data from individual slices were compared between jump and control groups of mice by analysis of variance (ANOVA) and post-hoc test. The precision of the repeated pQCT measurement at the mid-diaphyseal femur showed a coefficient of variation (CV) of less than 3% for all the parameters studied in this investigation.

Measurements of biochemical markers of bone turnover

Mouse C-telopeptide enzyme-linked immunosorbent assay (ELISA).
The C-telopeptide measurements were performed with a mouse C-telopeptide ELISA, described earlier [16]. The sensitivity of the ELISA was 0.3 ng/ml. The average within-assay CV was less than 7%; the average between-assay CV was less than 14%.

Mouse osteocalcin radioimmunoassay (RIA).
The osteocalcin measurements were performed with a mouse osteocalcin radioimmunoassay (RIA), described earlier [17]. The sensitivity of the RIA was 15 ng/ml. The average within-assay CV was less than 10%; the average between-assay CV was less than 15%.

Skeletal alkaline phosphatase assay.
Alkaline phosphatase was measured in serum by a kinetic method [7], using p-nitro-phenylphosphate (PNPP) as substrate and 15 mM L-phenylalanine to inhibit intestinal alkaline phosphatase. The L-phenylalanine inhibition assay exhibited intraassay (n = 10) and interassay (n = 8) CVs of 1.9% and 3.8%, respectively. The assay can detect less than 10 mU/ml of alkaline phosphatase in mouse serum.

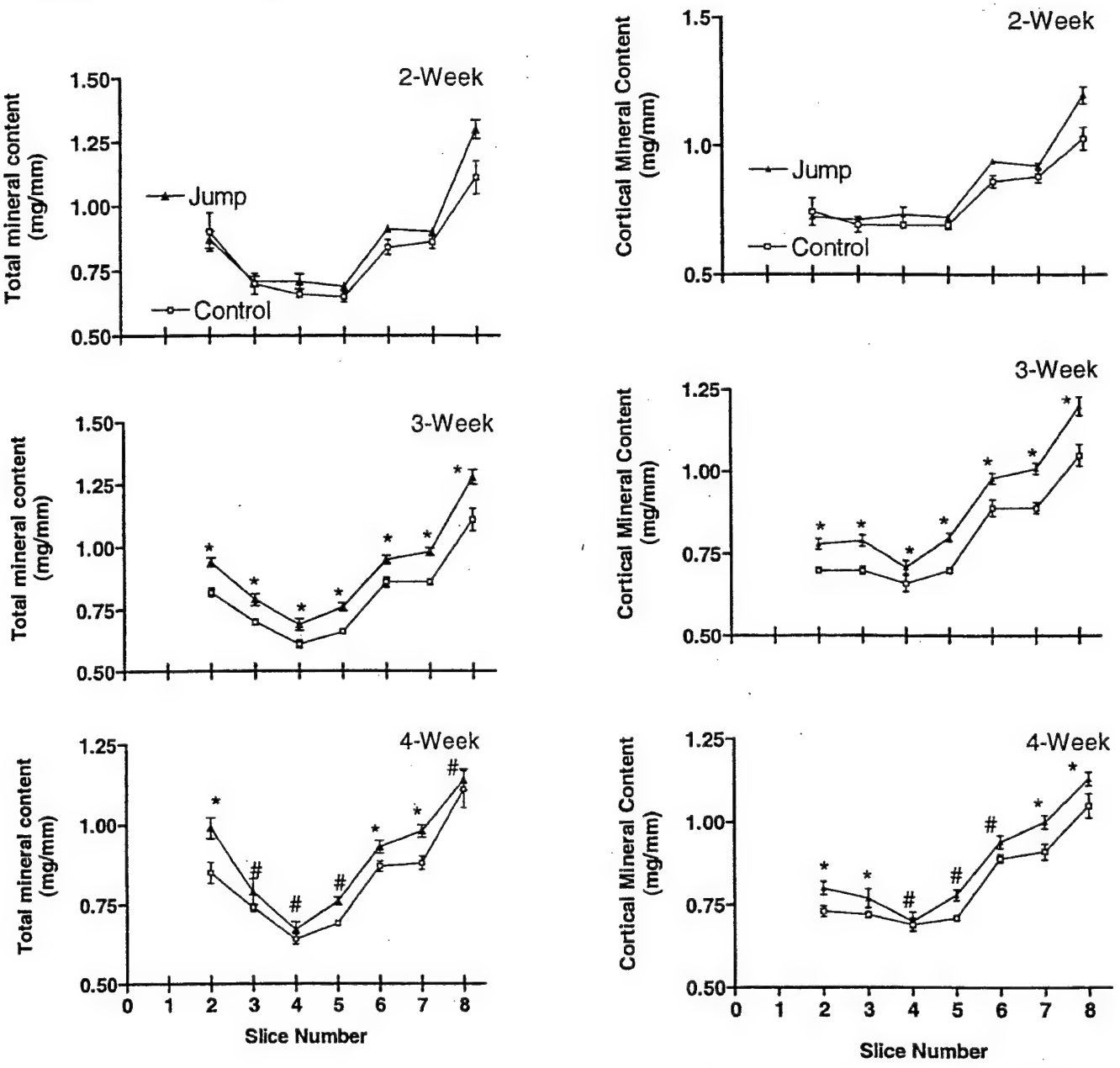

Fig. 1. Effects of 2, 3, and 4 weeks of jump exercise on cortical and total bone mineral content measured by peripheral quantitative computed tomography (pQCT) at nine different slices of the femur. Each slice was separated by 11% of the femur length, with slice 1 starting at the distal end. The data show (slices 1 and 9 were omitted due to large variation) that the increase in bone mineral content occurred throughout the length of the femur. Each data point represents the mean ± SEM ($n = 6$). The P value for both cortical and total bone mineral content of control vs jump group was <0.0001 by analysis of variance (ANOVA) for the 3-week and 4-week jump-exercise groups. The P values for comparison of individual slices in control and jump groups of mice by post-hoc tests are $^{*}P > 0.05$ and $^{*}P < 0.05$

Statistical analysis

Comparison of biochemical markers of bone metabolism between different groups was performed by the Mann-Whitney test (with a two-tailed P-value of <0.05 accepted as significant difference), because biochemical markers do not show normal distribution. All comparisons of pQCT femur structural data were performed on slice 5 by analysis of variance (ANOVA). ANOVA was used to reveal the differences among the control and the three jump-exercise groups. Post-hoc comparisons were performed by the Neuman-Keuls method and were used to determine the differences between specific means. All data are reported as means and standard deviations.

Results

As compared with the control group of mice, mice in the jump-exercise groups after 2, 3, and 4 weeks of exercise had no significant differences in body weight or longitudinal growth (length) of the femur, which was consistent with our previous findings [1]. The body weights were: 2-week control, 16.9 ± 1.1 g; 2-week jump group, 16.6 ± 0.7 g; 3-week control, 17.9 ± 1.1 g; 3-week jump group, 18.2 ± 0.5 g; 4-week control, 17.9 ± 1.0 g; and 4-week jump group, 17.5 ± 0.9 g. The femur lengths were: 2-week control, 13.7 ± 0.75 mm; 2-week jump group, 14.1 ± 0.18 mm; 3-week control, 14.4 ± 0.41 mm; 3-week jump group, 14.3 ± 0.19 mm; 4-week control, 14.0 ± 0.61; and 4-week jump group, 14.0 ± 0.20 mm.

Changes in mid-femur periosteal expansion and cross-sectional parameters are shown in Table 1. Periosteal circumference was increased by 3% ($P < 0.05$) after 3 weeks of exercise and remained higher in the 4 week exercise group (Table 1). Cortical area was increased by 11% ($P < 0.001$) after 3 weeks of exercise and was 8.4% higher than that in the controls in the 4-week exercise group ($P < 0.01$). Total area also increased, by 6.2% ($P < 0.01$), after 3 weeks of exercise, and remained elevated after 4 weeks. Cortical thickness was elevated by 8% ($P < 0.01$) and 5% (P, not significant) in the 3-week and 4-week exercise group, respectively. Although there was a time-related increase in endosteal circumference, the P values did not reach statistically significant levels.

Changes in total bone mineral density (BMD) and total and cortical bone mineral content (BMC) in the control and jump exercise groups are shown in Table 2. Changes for total and cortical BMC and total BMD between control and exercise groups did not reach significance for the 2-week exercise period. Maximum increases in total BMD and both total and cortical BMC were observed in the 3-week exercise group. Total BMD and total BMC were increased by 11.2% ($P < 0.01$) and 12.5% ($P < 0.01$), respectively, in the 3-week exercise group compared with the control group. Total BMC and total BMD values remained significantly elevated in the 4-week exercise group, and were 11% ($P < 0.01$) and 5% ($P < 0.05$), respectively, higher as compared with the control group, ANOVA values for the increase in total BMD and total BMC for treatment and weeks of exercise are shown in Table 3.

Figure 1 shows the plots for individual slices of total and cortical bone mineral content. The cortical and total BMC were significantly increased in the 3-week and

Table 1. Cross-sectional parameters and area of mid-shaft femur

	2-Week		3-Week		4-Week	
Parameters	Control	Jump	Control	Jump	Control	Jump
Periosteal circumference (mm)	4.23 ± 0.08	4.24 ± 0.052	4.22 ± 0.08	4.35 ± 0.083*	4.19 ± 0.04	4.33 ± 0.11*
Endosteal circumference (mm)	3.27 ± 0.06	3.23 ± 0.08	3.22 ± 0.06	3.27 ± 0.10	3.17 ± 0.06	3.25 ± 0.103
Cortical thickness (mm)	0.15 ± 0.01	0.16 ± 0.006	0.16 ± 0.003	0.17 ± 0.007**	0.16 ± 0.01	0.17 ± 0.005[a]
Total area (mm^2)	1.43 ± 0.05	1.43 ± 0.033	1.42 ± 0.05	1.50 ± 0.059**	1.40 ± 0.03	1.48 ± 0.075**
Cortical area (mm^2)	0.53 ± 0.03	0.60 ± 0.019	0.59 ± 0.013	0.66 ± 0.026**	0.59 ± 0.03	0.64 ± 0.028**

* $P < 0.05$; ** $P < 0.01$
Values are means ± SD
[a] NS, Not significant

Table 2. Bone mineral content and bone density parameters of mid-shaft femur

	2-Week		3-Week		4-Week	
Parameters	Control	Jump	Control	Jump	Control	Jump
Cortical mineral content (mg/mm)	0.69 ± 0.04	0.72 ± 0.02	0.71 ± 0.03	0.80 ± 0.02**	0.71 ± 0.014	0.78 ± 0.04*
Total mineral content (mg/mm)	0.65 ± 0.05	0.69 ± 0.03	0.68 ± 0.04	0.76 ± 0.04**	0.69 ± 0.02	0.76 ± 0.03**
Total bone mineral density (mg/cc)	409 ± 30	434 ± 6	425 ± 19	461 ± 8**	445 ± 13	465 ± 17*

* $P < 0.05$; ** $P < 0.01$
Values are means ± SD

Table 3. *P* values for analysis of variance (ANOVA) and Newman-Keuls post-hoc tests

	ANOVA Treatment	ANOVA Weeks	Post-hoc; week 2 vs week 3 or week 4	Post-hoc; week 3 vs week 4
Periosteal circumference at mid-femur (mm)	$P = 0.046$	NS	NS	NS
Total bone mineral content (mg/mm)	$P = 0.03$	$P = 0.01$	$P < 0.014$	NS
Total bone mineral density (mg/cc)	$P = 0.004$	$P = 0.03$	$P < 0.03$	NS

Table 4. Biochemical parameters in jump-exercise groups compared with control groups of mice

	2-Week		3-Week		4-Week	
Parameters	Control	Jump	Control	Jump	Control	Jump
Skeletal ALP (U/l)	152 ± 12	141 ± 34	175 ± 15	191 ± 24	164 ± 23	166 ± 23
Osteocalcin (ng/ml)	166 ± 32	168 ± 46	237 ± 60	200 ± 23	170 ± 58	141 ± 20
C-telopeptide (ng/ml)	4.4 ± 1.4	5.9 ± 1.8	5.2 ± 1.4	6.0 ± 1.7	6.2 ± 1.2	6.1 ± 1.8

Differences between control and jump groups were not significant
Values are means ± SD
ALP, Alkaline phosphatase

4-week jump groups, by ANOVA ($P < 0.0001$), as compared with the respective control groups of mice. The increases in cortical and total BMC in the 3-week jump group were statistically significant (by post-hoc test) for slices 2–8, as compared with the 3-week control group. Differences between the 4-week control and exercise groups for total BMC were not significant (by post-hoc test) for slices 3–5 and 8; similarly, differences in cortical BMC for slices 4–6 were not significant.

Although some parameters, such as subcortical content (the subcortical zone is mixed cortical and trabecular bone at the endosteal surface), cortical BMC, and total BMD showed a tendency to be lower in the 4-week exercise group as compared with the 3-week exercise group, these changes were not significant by ANOVA (Table 3).

Serum levels of skeletal alkaline phosphatase, osteocalcin, and C-telopeptide are shown in Table 4. None of the biochemical markers showed any significant differences between control and jump groups of mice at any time point. Data on the 4-week group were consistent with our earlier findings [1] showing that no systemic markers were elevated after 4 weeks of jump exercise.

Discussion

The bone formation response to a mechanical loading cycle of 20jumps/day for 4 weeks in the C57BL/6J mouse model has been well documented [1]. The elevated mid-femur bone mass and area after 4 weeks of jump exercise seen in the present study is consistent with earlier findings showing a similar response to jump exercise. However, we used a lower loading cycle, of 10jumps per day, compared with the previous study that used a loading cycle of 20jumps/day. We made this change because it has been recently reported that, in rats, a substantial bone formation response could be obtained in loading cycles of 10jumps/day, and the higher loading cycles resulted in only a marginal increase in bone response [15]. The lower loading cycle used in this study will also suit the QTL studies where large-scale screening may be required.

Our results indicate that there were slight increases in cortical area and bone mineral content (BMC) after 2 weeks of exercise, as compared with the control group of mice. However, these changes did not reach statistical significance. It could be assumed that the bone mass and area in response to jump exercise of 2 weeks duration were below the detection limits of the methods (pQCT) used in this study.

After 3 weeks of jump training, periosteal circumference, which is the most efficient means of measuring adaptation to exercise, clearly showed a significant increase in mid-femur structural parameters assessed by pQCT. Changes in bone mass and area after 3-week exercise were either greater than or comparable to those in the 4-week exercise group. However, periosteal circumference, bone density, and total and cortical BMC remained elevated in the 4-week jump group

when compared with the 4-week control group of mice. The 3% increase in periosteal circumference in the 4-week jump exercise group was consistent with our previous study [1], which showed about 2.2% higher periosteal perimeter after 4 weeks of jump exercise by histomorphometic analysis. Similarly, increases in cortical (8%) and total area (6%) after 4 weeks of exercise were consistent with the earlier findings observed at 4 weeks of exercise, which showed about 8% and 4% increases in cortical and total area, by histomorphometric analysis. Notably, we did not see any increase in bone mass and area after 4 weeks of jump exercise as compared with the 3-week jump-exercise group. It may be speculated that increases in bone density, mass, and area reached maximum levels after 3 weeks of loading and then the values plateaued, showing no more changes on further loading. However, in the absence of data on longer loading duration, this assumption needs confirmation.

A significant finding of this study was that the effect of jump exercise was most evident in the increase in total and cortical BMC measured by pQCT. Similar to other parameters, the highest increases in both cortical and total BMC were observed after 3 weeks of jump exercise. Our data on the cortical as well as the total BMC of nine slices (Fig. 1) indicate that the increase in BMC occurred throughout the entire length of the femur, and was not confined to the mid-shaft region of the femur. Comparisons of individual slices showed that the increases in total and cortical BMC were significant for the 3-week exercise group. In the 4-week exercise group, these differences did not reach the significance level for slices 3–6, mainly due to the larger variation in findings in the jump group, and also because the post-hoc test used to test statistical significance involved several variables, thus increasing the stringency of the test. A less conservative test indicated that the total and cortical BMC in most of the slices were both significantly higher in the jump groups as compared with the controls. This increase in the BMC of the entire length of the femur was particularly interesting, because earlier studies explored only a small area at a specific bone site [1–3]; thus, it was not clear whether the increase in bone mass was localized in the mid-shaft region or whether was spread throughout the length of the femur. Data presented in this study (Fig. 1) amply illustrate that the relative amount of bone mineral comprising the middle 10–12 mm of the diaphysis was consistently 9%–10% higher in the jump groups of mice as compared with the control mice.

Consistent with our earlier study [1], the endosteal circumference did not change significantly at any time point, including the 4 weeks of jump exercise. In addition, we did not observe any systemic response in bone turnover markers. Therefore, biochemical markers may not be useful in detecting early bone response to mechanical loading.

In conclusion, the present investigation showed that a significant increase in bone mass and area at our sampling site occurred after 3 weeks of jump exercise, and, as expected, these parameters remained elevated after 4 weeks of jump exercise. The results of this study show that a relatively small number of jumps/day and a short duration of jump exercise can achieve a detectable bone response; therefore, longer periods of exercise may not be necessary for bone hypertrophy to develop in mice. In addition, the data presented in this study indicate that pQCT measurements can be, effectively used to quantitatively determine changes in bone mass and area in response to the mechanical loading induced by jump exercise.

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